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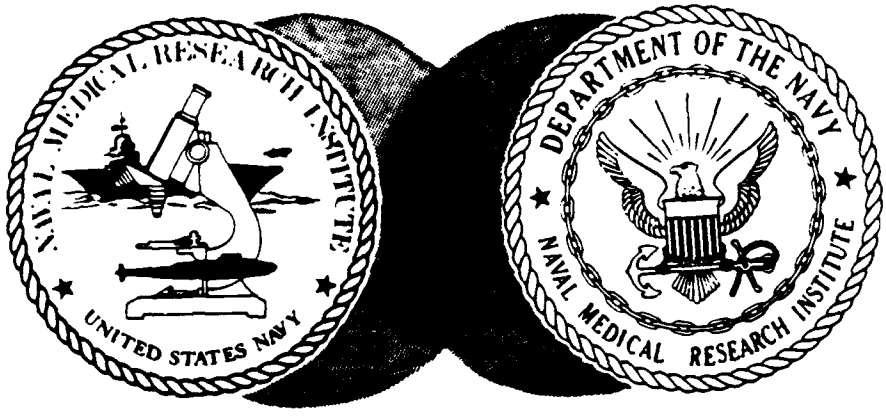

81-92

EFFECT OF FORMALIN TOXOIDING ON PSEUDOMONAS AERUGINOSA TOXIN A: BIOLOGICAL CHEMICAL AND IMMUNO-CHEMICAL STUDIES

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REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER: 81-92	2. GOVT ACCESSION NO.: A122532
3. RECIPIENT'S CATALOG NUMBER:	
4. TITLE *(and Subtitle)*: Effect of formalin toxoiding on Pseudomonas aeruginosa toxin A: Biological chemical and immunochemical studies	5. TYPE OF REPORT & PERIOD COVERED: Medical Research Progress Report
	6. PERFORMING ORG. REPORT NUMBER:
7. AUTHOR(s): Cryz,S.J.Jr., R.L. Friedman, O.R.Pavlovskis and B.H. Iglewski	8. CONTRACT OR GRANT NUMBER(s):
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Naval Medical Research Institute Bethesda, Maryland 20814	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: M0095.PN.002.5052 Report No. 3
11. CONTROLLING OFFICE NAME AND ADDRESS: Naval Medical Research & Development Command Bethesda, Maryland 20814	12. REPORT DATE: May 1981
	13. NUMBER OF PAGES: 10
14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*: Bureau of Medicine & Surgery Department of the Navy Washington, D.C. 20372	15. SECURITY CLASS. *(of this report)*: Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE:

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release and sale; distribution unlimited

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

published in: Infection and Immunity 32(2):759-768, May 1981

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Exotoxins; formaldehyde; formalin, mice

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

see reverse

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S/N 0102-LF-014-6601

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We investigated the effect of Formalin toxoiding on the biological, chemical, and immunological activities of *Pseudomonas aeruginosa* toxin A. Formalin treatment alone resulted in a 1,000-fold decrease in toxin-induced cell cytotoxicity and altered the antigenicity of the toxin A molecule without adversely affecting enzymatic activity. Competitive blocking experiments indicated that Formalin-mediated detoxification proceeded via alterations in a region of the toxin molecule other than the active site of the enzyme. The addition of lysine to Formalin-toxin mixtures not only increased the rate and extent of detoxification and antigenic alteration, but also completely destroyed enzymatic activity. The immunogenicities of different toxoids varied substantially. Upon dialysis and storage, Formalin-derived toxoids underwent partial toxic reversion, whereas a Formalin-lysine-derived toxoid did not. The sodium dodecyl sulfate-polyacrylamide gel patterns of Formalin- and Formalin-lysine-treated toxins indicated that these treatments caused the formation of a heterogenous group of high-molecular-weight species and produced substantial changes in the electrophoretic mobility of toxin A.

INFECTION AND IMMUNITY, May 1981, p. 759-768
0019-9567/81/050759-10$02.00/0
Vol. 32, No. 2

Effect of Formalin Toxoiding on *Pseudomonas aeruginosa* Toxin A: Biological, Chemical, and Immunochemical Studies

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We investigated the effect of Formalin toxoiding on the biological, chemical, and immunological activities of *Pseudomonas aeruginosa* toxin A. Formalin treatment alone resulted in a 1,000-fold decrease in toxin-induced cell cytotoxicity and altered the antigenicity of the toxin A molecule without adversely affecting enzymatic activity. Competitive blocking experiments indicated that Formalin-mediated detoxification proceeded via alterations in a region of the toxin molecule other than the active site of the enzyme. The addition of lysine to Formalin-toxin mixtures not only increased the rate and extent of detoxification and antigenic alteration, but also completely destroyed enzymatic activity. The immunogenicities of different toxoids varied substantially. Upon dialysis and storage, Formalin-derived toxoids underwent partial toxic reversion, whereas a Formalin-lysine-derived toxoid did not. The sodium dodecyl sulfate-polyacrylamide gel patterns of Formalin- and Formalin-lysine-treated toxins indicated that these treatments caused the formation of a heterogenous group of high-molecular-weight species and produced substantial changes in the electrophoretic mobility of toxin A.

Pseudomonas aeruginosa is an opportunistic pathogen which causes severe infections in compromised hosts (9, 10, 22, 29). Studies on the pathogenesis of *P. aeruginosa* infections have yielded information which suggests that an extracellular toxin (toxin A) may be an important virulence factor (25-27). Toxin A has been shown to be produced in vivo during infections (15, 28). Toxin A is known to be a potent inhibitor of eucaryotic protein synthesis (21, 25, 27), acting catalytically to transfer the adenosine 5'-diphosphate ribose moiety of nicotinamide adenine dinucleotide onto elongation factor II in a manner similar, if not identical, to the transfer mediated by diphtheria toxin (5, 11, 12).

The structure-activity relationship(s) of toxin A appears to be complex. Toxin A is believed to be released from bacterial cells predominantly in a proenzyme form (5, 17, 18, 20, 33). In vitro, full enzymatic activity is expressed only after reduction and denaturation, presumably through the exposure of the enzyme active site (5, 17, 18, 20, 33). Toxin A may be "activated" partially by repeated freezing and thawing (33). However, this results in the dissociation of the toxin molecule into two fragments, an enzymatically active fragment of 27,000 daltons (fragment a) and an enzymatically inactive fragment of 45,000 daltons (fragment b) (33). Enzymatically active fragments can also be produced by both chemical and proteolytic treatments (20).

Given the potentially important role that toxin A may play in *P. aeruginosa* infections, a number of studies have been directed toward producing an efficacious toxoid vaccine (1; S. H. Leppla, O. C. Martin, and O. R. Pavlovskis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B96, p. 24; M. Pollack and R. K. Prescott, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, B63, p. 26). Although toxin A has been detoxified by both Formalin and glutaraldehyde treatments, the nature of the chemically induced alteration(s) has not been studied in any detail. In this study we produced Formalin toxoids of toxin A under a variety of conditions. Various biological, chemical, and immunological characteristics of these toxoids were studied as a first step in the development of an efficacious toxoid vaccine.

MATERIALS AND METHODS

Toxin A. Toxin A was purified as previously described (13). The 50% lethal dose of our toxin preparation for 20-g Swiss-Webster mice was 0.20 μg when it was injected intraperitoneally. This toxin migrated as a single band on sodium dodecyl sulfate (SDS)-polyacrylamide slab gels (without prior heating at 100°C) and had a molecular weight of approximately 70,000.

Formalin treatment of toxin A. A modification of the procedure described by Linggood et al. (19) for the production of a Formalin diphtheria toxoid was used to detoxify toxin A. Purified toxin A was diluted in saline (pH 7.5) or saline containing 0.1 M lysine or 0.1 M proline to yield a final concentration of 1 mg of toxin per ml. Formalin (as a 37% formaldehyde solu-

tion) was added to a final concentration of 0.5% (vol/vol), and the pH was readjusted to 7.5. Solutions were filter sterilized and stored at 22°C in sterile polystyrene test tubes. Samples were withdrawn aseptically at different times, and each sample was divided into two portions; one of these portions was tested immediately in an assay, and the other was frozen at −70°C.

Radioimmunoassay for toxin A. The following procedures were used to prepare radiolabeled toxin A for use in radioimmunoassays; 1 mCi of carrier-free $Na^{125}I$ (Amersham Corp., Arlington Heights, Ill.) was added to 50 μg of highly purified toxin A and 10 μg of lactoperoxidase in 1 ml of phosphate-buffered saline (pH 7.2). The enzymatic iodination reaction was initiated by adding 25 μl of 0.03% hydrogen peroxide (34). After a 5-min incubation at 22°C, an additional 25-μl portion of 0.03% H_2O_2 was added, and the reaction was allowed to continue for 5 min. This labeled preparation was dialyzed for 18 h at 4°C in 1 liter of phosphate-buffered saline. The dialyzed sample was applied at 4°C to a Sephadex G-100 column (2.2 by 30 cm; bed volume, 88 ml) equilibrated with phosphate-buffered saline containing 1 mg of bovine serum albumin per ml and then eluted with the same buffer. Fractions containing the radiolabeled toxin were pooled. Sodium azide was added to a concentration of 0.02%, and the preparations were stored at 4°C. Labeled samples routinely possessed a specific activity of more than 2 μCi/μg of toxin protein and were more than 90% immunoprecipitable in the presence of excess antitoxin (see below).

IgG Sorb (Enzyme Center, Boston, Mass.), a Formalin-fixed preparation of a protein A-bearing strain of *Staphylococcus aureus*, was used as a particulate immunoadsorbent for immune complexes containing immunoglobulin G (14). Samples were supplied lyophilized, reconstituted to 10% (vol/vol) in NET buffer [150 mM NaCl, 5 mM ethylenediaminetetraacetate, 50 M tris(hydroxymethyl)aminomethane, pH 7.4], and stored at 4°C. Immediately before use in radioimmunoassays, cells were pelleted by centrifugation at 4,000 × *g* for 15 min at 4°C. Cells were suspended to 10% (vol/vol) in NET buffer containing 0.5% Nonidet P-40 (Particle Data Laboratories, Elmhurst, Ill.) and then incubated for 10 min at 22°C. The cells were then washed once with NET buffer containing 0.05% Nonidet P-40 and finally suspended to 10% (vol/vol) in assay buffer (NET buffer containing 0.05% Nonidet P-40 and 1 mg of bovine serum albumin per ml).

The antitoxin used in the radioimmunoassays was raised in rabbits by multiple injections of highly purified toxin A suspended in an equal volume of Freund complete adjuvant, as previously described (3). Booster doses contained toxin suspended in an equal volume of Freund incomplete adjuvant.

Radioimmunoassays were carried out in final volumes of 0.5 ml in polystyrene test tubes (10 by 75 mm). Assay buffer was used as the diluent for all reagents. Reagents were added in the following sequence: (i) assay buffer, (ii) approximately 10,000 cpm of ^{125}I-labeled toxin in 10 μl, (iii) nonradioactive antigen (5 to 100 μl/assay), and (iv) 0.01 μl of rabbit antitoxin, which was sufficient to immunoprecipitate between 50 and 60% of the total counts in the absence of competing nonradioactive antigen. Assay mixtures were incubated for 15 min at 22°C. To each assay 50 μl of IgG Sorb was added, and the samples were incubated for an additional 10 min at 22°C. Immune complexes were collected by centrifugation at 3,000 × *g* for 15 min at 4°C and then washed twice with 1 ml of assay buffer, and the final pellets were counted with a Beckman Biogamma counter. The data from the radioimmunoassays were expressed as described by Ekins (8).

Polyacrylamide gel electrophoresis. SDS-polyacrylamide slab gel electrophoresis was performed by the method of Laemmli (16). Polyacrylamide gels (composed of a 7.5% resolving gel and a 4.5% stacking gel) were cast at a thickness of 0.75 mm. Protein samples (0.5 to 10 μg) were boiled for 5 min in the presence of 5% mercaptoethanol and 1.25% SDS immediately before application to the gels. Gels were electrophoresed at room temperature at a constant power of 1.2 W/gel in the buffer system described by Laemmli (16). Gels were stained with 0.05% Coomassie brilliant blue R-250 in 25% isopropanol–10% acetic acid and destained with 10% acetic acid. We used the following molecular weight standards: phosphorylase b, bovine serum albumin, pyruvate kinase, ovalbumin, lactate dehydrogenase, and carbonic anhydrase. Diphtheria toxin was purchased from Connaught Laboratories, Toronto, Canada, and further purified as previously described (7).

Enzyme assay and neutralization by antibody. The adenosine 5′-diphosphate ribose transferase activity of toxin A was measured as described previously (13). Samples containing 100 ng of toxin protein (sufficient, after activation, to produce 60% saturation in a 5-min assay) were tested before and after activation with urea and dithiothreitol (13). Enzyme neutralization assays were performed in the presence of 0.5% normal rabbit serum. Toxin (100 ng; treated with urea and dithiothreitol) in 10 μl was preincubated with an equivalent amount of normal rabbit serum or various antisera for 10 min at 37°C. The mixtures were chilled rapidly on ice and then assayed as described above.

Cytotoxicity assay and neutralization by antibody. The Chinese hamster ovary (CHO) cell assay for toxin-induced cytotoxicity was performed as previously described (13). The minimal cytotoxic dose (MCD) was defined as the smallest quantity of toxin or toxoid which prevented a colorimetric change in the growth medium after 72 h of incubation at 37°C in 5% CO_2. With the purified toxin A used in this study, the MCD was 0.01 ng of toxin per 2×10^4 CHO cells. The cytotoxic neutralization titers of antisera were determined as follows. To each well of a microtiter plate, 25 μl of medium, 5 MCDs (0.05 ng) of purified toxin A, and 10 μl of antiserum were added. The mixtures were incubated for 15 min at 37°C. CHO cells were added, and the plates were scored after an additional 72 h of incubation. Control wells containing cells plus normal rabbit serum and 5 MCDs of toxin and control wells containing cells plus antiserum (no toxin) were included in each plate. All assays were done in duplicate.

Production of antisera. Antitoxoids were produced in rabbits by using the following immunization schedule. Each New Zealand white rabbit was injected subcutaneously, intramuscularly, and in the rear foot-

pads with a total of 50 μg of toxoid in saline (pH 7.4) mixed with an equal volume of Freund complete adjuvant. Each rabbit was boosted by using the same schedule and 50 μg of antigen mixed with an equal amount of Freund incomplete adjuvant at 10 and 17 days after the initial immunization. Animals were exsanguinated 10 days after the final injection series, and the sera were collected and stored at −20°C. Mouse antitoxoids were prepared in female Swiss white mice (NIH/NmC, cv strain) weighing approximately 20 g. For immunizations without adjuvant, the initial dose was given intraveneously in 100 μl of saline containing 9 μg of toxoid. Subsequent doses (9 μg of toxoid in 100 μl of saline) were given 24, 37, and 44 days after the initial injection. Sera were obtained 7, 47, and 68 days after the initial injection. *N*-acetyl muramyl-L-alanyl-D-isoglutamine was used as an adjuvant. Doses consisted of 50 μg of *N*-acetyl muramyl-L-alanyl-D-isoglutamine and 10 μg of toxoid in 200 μl of saline. The initial dose was given intravenously, and the following injections were given intramuscularly 14, 26, and 42 days after the first immunization. Animals were bled 9, 35, 49, and 70 days after the initial immunization. Sera were stored at −20°C.

Stability of toxoids. Toxoids were tested for the ability to regain toxicity once Formalin was removed. Toxoids were diluted with saline (pH 7.4) to yield a final protein concentration of 100 μg/ml. The diluted toxoids were then dialyzed against 2,000 volumes of phosphate-buffered saline for 18 h at 4°C and sterilized by membrane filtration (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). Toxoids were then placed in sterile test tubes at 4, 22, and 37°C. Samples were withdrawn at different times, and the MCD for CHO cells was determined.

RESULTS

Effects of Formalin and amino acids on toxin-induced cytotoxicity. Purified toxin A was incubated at 22°C in the presence of 0.5% Formalin with or without amino acids, as described above. Toxin alone was incubated in sterile saline at 22°C, and this was used as a control in all experiments. At intervals of 24, 48, 72, 120, and 240 h, treated samples and controls (consisting of toxin incubated alone and freshly thawed toxin) were withdrawn and assayed. Figure 1 shows the effects which various treatments had on toxin-induced CHO cell cytotoxicity. Toxin incubated for up to 240 h alone or in the presence of lysine or proline showed no loss of cytotoxicity. Incubation with Formalin alone or with Formalin plus proline resulted in a progressive loss of cytotoxicity over a 72-h period. By 72 h these two preparations had undergone a 1,000-fold reduction in cytotoxicity. Toxin incubated with Formalin plus lysine showed progressive 100-fold decreases in toxicity after 24 and 48 h of incubation and a further 10-fold decrease between 48 and 72 h. By 72 h the Formalin-lysine preparation had undergone a 100,000-fold reduction in cytotoxicity. These results were extended by testing the mouse toxicity of a preparation (Formalin-lysine preparation at 48 h) which had undergone a substantial decrease in cytotoxicity. The 50% lethal dose of the untreated toxin A preparation was 0.2 μg/22-g mouse when it was injected intraperitoneally. In contrast, six animals injected intraperitoneally with 50 μg (250 50% lethal dose equivalents) of the Formalin-lysine preparation survived. These mice were sacrificed after 12 days, and the liver, spleen, kidneys, lungs, and heart of each were examined for histopathological alterations. No pathological changes were observed. The above-described results demonstrated that Formalin-mediated detoxification of toxin A was a time-dependent process and that both the rate and extent of detoxification could be increased markedly by adding lysine to Formalin-toxin mixtures.

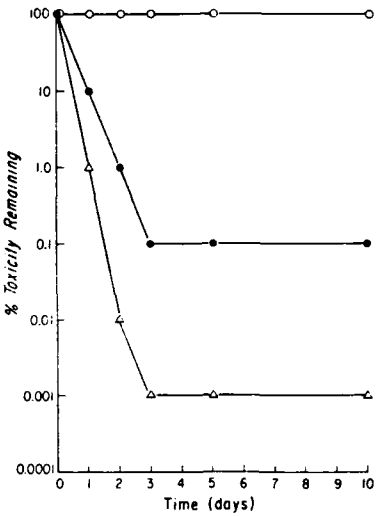

FIG. 1. *Residual CHO cell cytotoxicity of toxin A after incubation with Formalin or amino acids or both. Toxicity was calculated as the MCD of each preparation per 2 × 10⁴ CHO cells. The MCD for native toxin A was 0.01 ng. Assays were performed in duplicate. Symbols: ○, native toxin (toxin incubated alone in sterile saline and toxin incubated in the presence of 0.1 M lysine or 0.1 M proline); ●, toxin incubated with Formalin alone or with Formalin plus proline; △, toxin incubated with Formalin plus lysine.*

Effects of toxoiding on the enzymatic activity of toxin A. The effect which each toxoiding procedure had on the adenosine 5′-diphosphate ribose transferase activity of toxin A was determined by testing samples for enzymatic activity before (nonactivated) and after (activated) urea-dithiothreitol activation. Samples treated with Formalin plus lysine for 24 h showed a loss in enzymatic activity of approximately 75% (data not shown). The enzymatic

activities of other preparations at 24 h remained unaltered. By 48 h, the Formalin-lysine treatment had completely destroyed the enzymatic activity of toxin A (Fig. 2). Also noted at 48 h was a decrease of approximately 33% in the urea-dithiothreitol-potentiated activity of toxin treated with Formalin plus proline. We observed no further decrease in the enzymatic activity of toxin after continued incubation. Toxin incubated alone or in the presence of lysine or proline showed no loss of enzymatic activity. Incubation of toxin with Formalin alone or with Formalin plus proline for 48 h resulted in partial activation of the enzymatic activity (Fig. 2). We found that Formalin-mediated activation was a time-dependent process, with activation maximal by 120 h (Fig. 3). In contrast, nonactivated samples of toxin incubated alone at 22°C for up to 240 h expressed less then 1% of the enzymatic activity of urea-dithiothreitol-potentiated toxin A (Fig. 3).

Binding of toxoids to CHO cells. The above-described results showed that Formalin-mediated detoxification of toxin A was not due solely to the destruction of enzymatic activity and suggested that the preparations tested were nontoxic due to an inability to bind to the membranes of sensitive cells. The ability of each toxoid to block toxin-induced CHO cell cytotoxicity was used as an indication of the ability of the toxoid to bind to sensitive cell membranes. Toxoids were titrated against 2 MCDs of toxin with toxin and toxoids added simultaneously. CHO cell assays were performed as described above. Even at a ratio of toxoid to toxin of 500:1, there was no blocking effect on cell intoxication. These results indicated that the toxoid preparations tested were altered in a region which was essential for binding to sensitive cells.

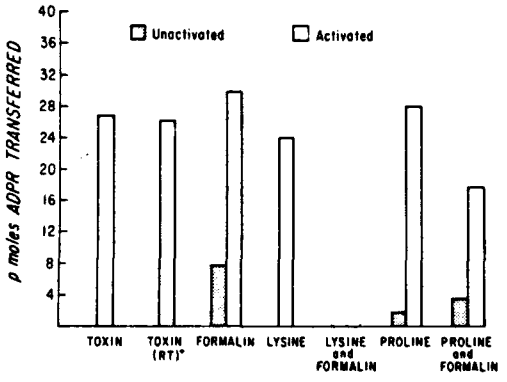

FIG. 2. *Residual enzymatic activity of toxin A after incubation with Formalin or amino acids or both for 48 h. Samples were tested before (nonactivated) and after (activated) treatment with urea plus dithiothreitol. Data represent averages of duplicate samples. The entire experiment was repeated twice with comparable results. (RT)*, Toxin incubated alone in sterile saline at 22°C; ADPR, adenosine 5'-diphosphate ribose.*

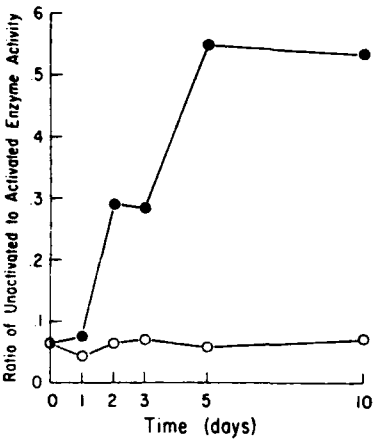

FIG. 3. *Time-dependent activation of toxin A by Formalin. Ratios of nonactivated enzyme activity to activated enzyme activity were obtained in the following manner. The amount of ^{14}C labeled adenosine 5'-diphosphate ribose transferred into acid-insoluble material by nonactivated Formalin-treated samples was divided by the amount transferred by activated samples (treated with urea plus dithiothreitol). Zero time values represent the average values given by untreated toxin at each time point. The data represent the average values of duplicate samples. Symbols: ●, toxin treated with Formalin alone and incubated at 22°C; and ○, toxin in sterile saline incubated at 22°C.*

Stability of toxoids to reversion. Three toxoids were tested for toxic reversion upon removal of Formalin as a function of time and temperature. Toxoids prepared by treatment with Formalin alone for either 72 or 240 h did not revert when stored for up to 21 days at 4°C. However, when they were stored at 22 or 37°C, a 10-fold increase in cytotoxicity was observed by 7 days. Continued incubation did not result in further reversion. Toxoid produced by treatment with Formalin plus lysine for 48 h did not show detectable evidence of reversion at any of the three temperatures tested, even when it was stored for up to 45 days.

Antigenic alterations induced by Formalin treatment. A liquid-phase radioimmunoassay was developed to monitor antigenic changes induced in the toxin molecule by toxoiding. Controls demonstrated that toxin incubated alone or in the presence of proline or lysine was not altered antigenically, as evidenced by a linear displacement of labeled antigen from im-

mune complexes with increasing quantities of nonradioactive antigen (data not shown). Incubation of toxin with Formalin alone resulted in a progressive loss of native antigenicity (Fig. 4). The curves generated at all time points were composed of two linear phases of differing slopes. Greater quantities of labeled antigen were displaced per unit weight of nonradioactive antigen in the first phase than in the second phase. These findings suggested that certain antigenic determinants on the toxin molecule were altered preferentially by Formalin due either to their increased availability or to reactivity with Formalin. Even after 240 h of incubation in the presence of Formalin, substantial levels of cross-reactivity remained (Fig. 4).

The addition of lysine to Formalin-toxin mixtures greatly changed both the rate and the extent to which the toxin molecules were altered antigenically (Fig. 5). The curves generated by samples incubated for up to 120 h were biphasic, as observed with Formalin treatment (Fig. 5). However, the antigenicity of Formalin-lysine-treated toxin continued to decrease with time, so that by 240 h no detectable cross-reactivity remained (Fig. 5). These results were confirmed by gel diffusion analysis with antitoxin. Whereas toxin treated with Formalin for up to 240 h gave a line of identity with toxin, toxin treated with Formalin plus lysine for only 48 h yielded a line of partial identity (Fig. 6). Toxin treated with Formalin plus lysine for 240 h showed no reactivity with antitoxin (data not shown).

Electrophoretic mobility of toxoids. In addition to blocking amino groups, Formalin and glutaraldehyde have been shown to form inter- and intramolecular cross-links in proteins. The alterations in the toxicities, antigenicities, and enzymatic activities of Formalin-treated samples of toxin A strongly suggested that Formalin treatment may have caused substantial structural changes within the toxin molecule. This possibility was investigated by analyzing the SDS-polyacrylamide gel patterns of various Formalin-treated preparations (Fig. 7 and 8). An excess of protein (10 μg/well) was applied to the gel shown in Fig. 7 so that minor changes in the resulting gel profiles could be analyzed better. When treated with SDS and 2-mercaptoethanol without heating before electrophoresis, purified toxin A migrated as a single band, as previously reported by us and others (5, 13, 17, 20). How-

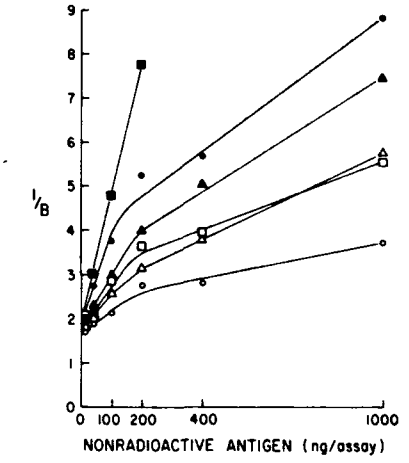

FIG. 4. *Antitoxin binding by toxin treated with Formalin alone. B represents the fraction of total radiolabeled antigen immunoprecipitated. Toxin was treated with Formalin for 24 h (○), 48 h (△), 72 h (□), 120 h (▲), or 240 h (●), or toxin was incubated alone for 240 h (■).*

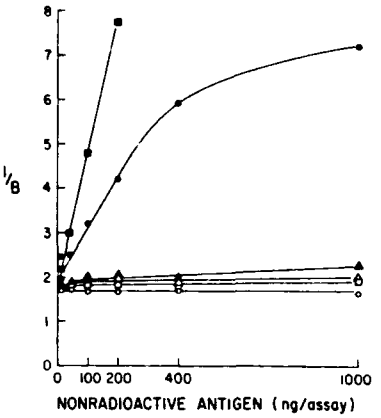

FIG. 5. *Antitoxin binding by toxin treated with Formalin plus lysine. B represents the fraction of radiolabeled antigen immunoprecipitated. Toxin was treated with Formalin plus lysine for 24 h (○), 48 h (△), 72 h (□), 120 h (▲), or 240 h (●), or toxin was incubated alone for 240 h (■).*

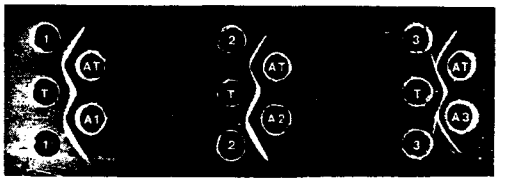

FIG. 6. *Gel immunodiffusion patterns comparing reactivities of various toxoid preparations with homologous rabbit antitoxoid and rabbit antitoxin. Antigen concentrations were 100 μg/ml. Well T, Toxin; well AT, antitoxin; well 1, toxin treated with Formalin for 72 h; well 2, toxin treated with Formalin for 240 h; well 3, toxin treated with Formalin plus lysine for 48 h; wells A1, A2, and A3, antitoxoids raised against the appropriate antigens.*

ever, heating toxin at 100°C for 5 min in the presence of SDS and high concentrations (5%) of 2-mercaptoethanol resulted in the generation of multiple peptides having lower molecular weights than toxin A (Fig. 7, lane 7). These peptides could be visualized only on overloaded SDS slab gels. Patterns were indistinguishable for native toxin and toxin incubated alone or in the presence of proline or lysine for 24 h (Fig. 7, lanes 1, 3, 6, and 7). This was true even when samples were incubated for up to 240 h (data not shown). We observed marked changes in the gel patterns of samples incubated with Formalin plus proline, Formalin plus lysine, or Formalin alone (Fig. 7, lanes 2, 4, and 5, respectively). The smallest of the heat-generated peptides were absent in these preparations. In the Formalin-lysine and Formalin preparations we also observed the formation of a heterogeneous population of molecules with molecular weights which were larger than the molecular weight of untreated toxin (Fig. 7, arrows). By 240 h, no heat-generated peptides could be observed in the Formalin-lysine-treated toxin, and the ratios of high-molecular-weight species to native toxin were increased substantially (data not shown).

FIG. 7. *SDS-polyacrylamide slab gel electrophoresis of toxin treated with Formalin or amino acids or both for 24 h at 22°C. Samples (10 μg) were heated at 100°C for 5 min in the presence of 1.25% SDS and 5% mercaptoethanol before application to the gel. Lane 1, Toxin treated with proline alone; lane 2, toxin treated with Formalin plus proline; lane 3, toxin treated with lysine alone; lane 4, toxin treated with Formalin plus lysine; lane 5, toxin treated with Formalin alone; lane 6, toxin incubated alone; lane 7, untreated toxin. The arrows indicate molecular weight species which were larger than native toxin.*

The structural alterations induced in the toxin molecule by treatment with Formalin alone or Formalin plus lysine were studied over a 240-h period. An appropriate quantity of protein (2 μg/well) was used to facilitate the detection of slight changes in the mobility of native toxin (Fig. 8). By 24 h the gel pattern produced by toxin treated with Formalin alone was altered noticeably (it was slightly more diffuse than the gel pattern of toxin incubated alone for 24 h). This phenomenon became more pronounced with time. However, the electrophoretic mobil-

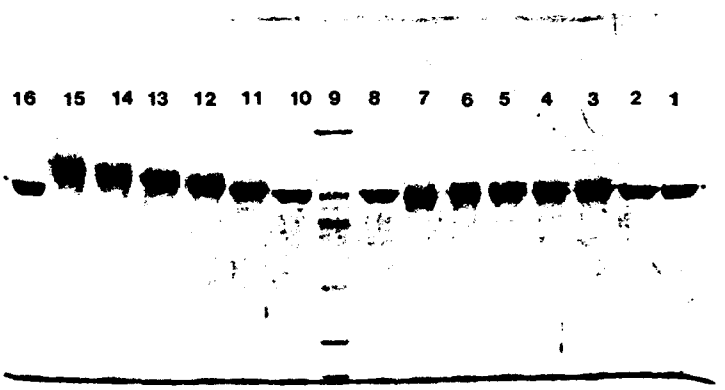

FIG. 8. *SDS-polyacrylamide slab gel electrophoresis of toxin treated with Formalin alone or with Formalin plus lysine for different times. Samples (2 μg) were heated at 100°C in the presence of 1.25% SDS and 5% mercaptoethanol before application to the gel. Lanes 1, 8, 10, and 16, untreated toxin; lane 2, toxin incubated alone for 240 h; lanes 3 through 7, toxin treated with Formalin alone for 24, 48, 72, 120, and 240 h, respectively; lane 9, molecular weight standards, including (from top to bottom) phosphorylase (molecular weight, 92,000), bovine serum albumin (68,000), diphtheria toxin (60,000), pyruvate kinase (57,000), ovalbumin (45,000), lactate dehydrogenase (36,000), and carbonic anydrase (30,000); lanes 11 through 15, toxin treated with Formalin plus lysine for 24, 48, 72, 120, and 240 h, respectively.*

ity of native toxin did not appear to change. The addition of lysine to Formalin-toxin mixtures resulted in a dramatic alteration both in the configuration and in the migration of the toxin band (Fig. 8, wells 11 through 15). Such changes could be detected after 24 h, were time dependent, and were more pronounced with increasing length of incubation.

Characteristics of antitoxin and antitoxoids. The immunogenicities of three toxoids were tested in rabbits and compared with the immune response elicited when native toxin was used as an immunogen. Two rabbits were immunized with each toxoid, as described above. The data shown below are from one representative animal from each group. Comparable results were obtained from each of the rabbits immunized with particular antigen. Toxin-binding capacity was defined as the smallest quantity of toxin resulting in a displacement of labeled antigen (toxin) from immune complexes (Fig. 9, arrows). These assays were performed in the presence of twice as much antiserum as necessary to give maximal precipitation of labeled toxin in the absence of competing antigen. Antitoxin and antitoxoid 1 (raised against toxin treated with Formalin for 72 h) had comparable binding capacities (450 and 420 μg of toxin per ml of serum, respectively). Antitoxoid 2 (raised against toxin treated with Formalin for 240 h) possessed the highest binding capacity (1,200 μg of toxin per ml of serum), whereas antitoxoid 3 (raised against toxin treated with Formalin plus lysine for 48 h) had the lowest binding capacity (70 μg/ml of serum).

Enzyme-neutralizing abilities of various antisera. Although the experiment described above demonstrated different titers of toxin precipitating antibody in the four antisera, it did not differentiate among antibody populations directed against antigenic determinants located at different regions on the toxin molecule. Since toxin A presumably is composed of two domains, only one of which is responsible for its enzymatic activity (33), it was of interest to determine the abilities of the toxoids to elicit enzyme-neutralizing antibody (Fig. 10). Antitoxoid 3 was ineffective at neutralizing enzymatic activity, completely inhibiting activity only at the highest concentration of antiserum tested (1:2 dilution). Enzyme-neutralizing ability was almost nonexistent at a 1:16 dilution. Antitoxin and antitoxoid 1 had nearly identical enzyme-neutralizing capacities; they still possessed nearly 50% of their activities at dilutions of 1:16 and 1:32, respectively. Antitoxoid 2 contained the highest titer of enzyme-neutralizing antibody, inhibiting enzymatic activity by more than 90% at a dilution of 1:64 and still possessing substantial enzyme-neutralizing activity at a dilution of 1:256. Based on the above-described results, antitoxoid 2 was 16 times more effective at neutralizing the adenosine 5′-diphosphate ribose transferase activity of toxin than antitoxoid 3 and at least 4 times as effective as antitoxoid 1 or antitoxin.

The CHO cell cytotoxicity-neutralizing titers (expressed as the reciprocal of the highest dilution of serum which completely neutralized 5 MCDs of toxin A) for antitoxin and antitoxoids 1, 2, and 3 were 512, 1,024, 2,048, and 256, re-

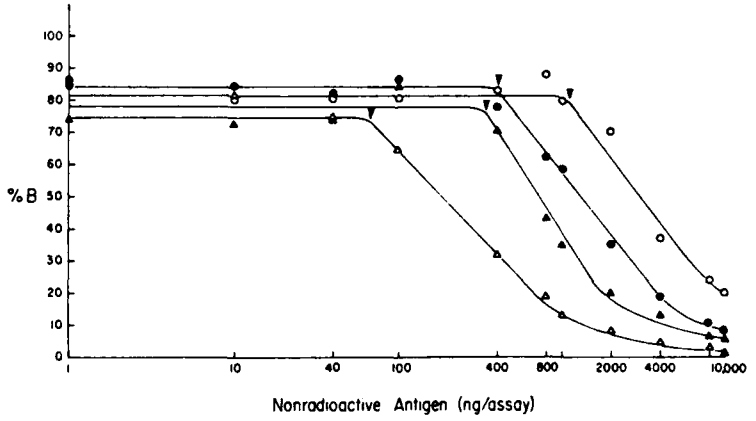

FIG. 9. *Toxin-binding capacities of rabbit antitoxin and antitoxoids. Each antiserum was titrated initially to determine the minimum quantity of antiserum needed to immunoprecipitate a maximal percentage of labeled toxin. Binding capacity determinations were carried out in twice this amount of antiserum. The arrowheads at the inflection points of the curves indicate the smallest concentrations of cold antigen which caused a reduction in the amount of radiolabeled antigen immunoprecipitated. Symbols: ▲, antitoxin; ●, antitoxoid 1 (toxin treated with Formalin for 72 h); ○, antitoxoid 2 (toxin treated with Formalin for 240 h); △, antitoxoid 3 (toxin treated with Formalin plus lysine for 48 h). % B, Percent binding.*

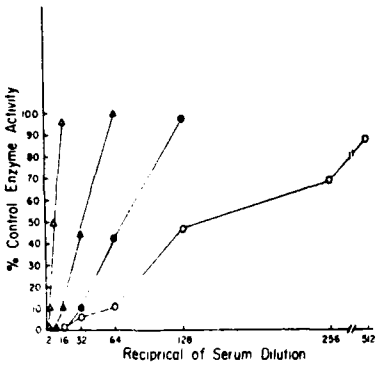

FIG. 10. *Enzyme neutralization by various antisera. Symbols: ▲, antitoxin; ●, antitoxoid 1 (toxin treated with Formalin for 72 h); ○, antitoxoid 2 (toxin treated with Formalin for 240 h); △, antitoxoid 3 (toxin treated with Formalin plus lysine for 48 h).*

spectively. Thus, in contrast to the differences observed between antitoxoids 2 and 3 in toxin-binding capacity (17-fold) and in enzyme neutralization (16-fold), we observed a difference of only 2-fold between these two toxoids in cell cytotoxicity titer.

The above-described results were extended by immunizing another animal species (mice) and comparing the immunogenicities of toxoids with and without adjuvant. Two toxoid preparations (Formalin treated for 240 h and Formalin-lysine treated for 48 h) were tested in mice (Table 1). These two preparations elicited comparable neutralizing titers when they were used with adjuvant (mean titers, 192 and 162). Although adjuvant resulted in only a modest increase in titer with the Formalin preparation, the antibody response with the Formalin-lysine preparation was approximately 40-fold greater when adjuvant was used. Furthermore, the sera from three of eight mice immunized with the Formalin-lysine preparation without adjuvant had no detectable CHO cell cytotoxicity-neutralizing titers.

DISCUSSION

Formalin has been used as a detoxifying agent for numerous bacterial toxins. Tetanus and diphtheria toxoid vaccines have been instrumental in the control of these two toxicoses, in which potent toxins are known to play central roles in the pathogenesis of these diseases (4, 32). Several studies have suggested that toxin A may play a role in the pathogenesis of *P. aeruginosa* infections (25–27). Combined with the high mortality rate of certain *P. aeruginosa* infections even in the fact of intensive antibiotic therapy, these studies have generated considerable interest in the development of a safe and efficacious toxin A vaccine. Although several studies have described the detoxification of toxin A by Formalin treatment (1; M. Pollack and R. K. Prescott, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, B63, p. 26), the mechanism(s) by which Formalin reduces toxicity and the effect of Formalin on the antigenicity and immunogenicity of the toxin molecule were not studied in detail. We have extended these studies by monitoring Formalin-induced immunochemical and structural alterations of the toxin molecule in vitro.

Our data indicate that Formalin-mediated detoxification of toxin A is not due solely to a loss of enzymatic activity (Fig. 1 and 2). Treatment of toxin A with Formalin alone resulted in a 1,000-fold decrease in cytotoxicity and yet had no deleterious effect on enzymatic activity. These results suggest that Formalin induces structural alterations in a region of the toxin A molecule which is essential for cytotoxicity but which is distinct from the site required for enzyme activity. The inability of formol toxoids to block toxin-induced CHO cell cytotoxicity indicates that the altered portion of the toxin molecule functions in the binding of toxin to sensitive cells. Similar findings have been reported for Formol diphtheria toxoid (6, 24).

In contrast to treatment of toxin A with Formalin alone, the addition of lysine to Formalin-toxin mixtures completely destroyed enzymatic activity within 48 h. Conformational changes within the toxin A molecule are believed to be essential for expression of enzymatic activity (5, 17, 20, 33). The loss of activity caused by Formalin-lysine treatment may be explained by the production of a stabilized molecule unable to

TABLE 1. *Immunogenicities of toxoids with and without adjuvant*[a]

Immunogen[b]	CHO cell cytotoxicity-neutralizing titer[c]	
	With adjuvant	Without adjuvant
Toxin + Formalin (240 h)	192 (70.1)	117 (44.2)
Toxin + Formalin-lysine (48 h)	162 (38.7)	3.5 (1.2)

[a] Groups of six mice were used for immunizations with adjuvant. Groups of eight mice were used for immunizations without adjuvant.

[b] Immunization schedules were as described in the text.

[c] Titer is expressed as the mean of the reciprocal of the highest dilution of serum sufficient to neutralize 5 MCDs of toxin, as determined by colorimetric change. Numbers in parenthesis are standard errors of the mean.

undergo such rearrangements due to the formation of inter- or intramolecular cross-links. Previous studies have demonstrated that diphtheria toxoid produced by Formalin-lysine treatment was far more resistant to toxic reversion than a toxoid produced by treatment with Formalin alone due, in part, to the incorporation of lysine into the toxin molecule (19, 30). We obtained similar results with toxin A, suggesting that Formalin-lysine treatment of toxin A resulted in a more stabilized molecule than treatment with Formalin alone.

The antigenicity of toxin A was altered by Formalin toxoiding (Fig. 4 through 6). Treatment of toxin A with Formalin alone resulted in a time-dependent destruction of some antigenic determinants, as evidenced by the non-linearity and decreased slopes of the competition curves. We interpret these results to mean that certain antigenic determinants are more sensitive to Formalin alteration than others. However, since large quantities of Formalin-derived toxoid were capable of almost completely blocking the immunoprecipitation of labeled toxin and a reaction of complete identity was found by gel immunodiffusion (Fig. 6), none of the immunodominant antigenic determinants of toxin A was destroyed quantitatively by treatment with Formalin alone. In contrast, treatment with Formalin plus lysine rapidly altered the antigenicity of the toxin molecule. After 48 h of incubation, the Formalin-lysine preparation was altered markedly, as evidenced by minimally detectable levels of competition in the radioimmunoassay (Fig. 6) and by a reaction of incomplete identity when the preparation was tested by gel immunodiffusion against antitoxin. After 240 h of Formalin-lysine treatment, the toxin molecule was altered to such an extent that it displayed no detectable activity in the radioimmunoassay. The sensitivity of toxin A to Formalin-lysine toxoiding differs dramatically from the sensitivity of diphtheria toxin. Bazaral et al. (2) demonstrated that, as determined by the radioimmunoassay, the antigenicity of diphtheria toxin treated with Formalin plus lysine for 40 h was not altered detectably. Results described elsewhere (7a) demonstrated that prolonged treatment (30 days) of diphtheria toxin with Formalin plus lysine did result in the preferential destruction of some antigenic determinants of the toxin molecule, but not in a quantitative manner. Similar preferential alterations were found with toxin A, but at a much accelerated rate.

The immunogenicities and stabilities of three toxoids (Formalin treated for 72 h, Formalin treated for 240 h, and Formalin-lysine treated for 48 h) depended upon the method of production (Fig. 9 and 10 and Table 1). Treatment of toxin with Formalin alone yielded preparations which were good immunogens in both mice and rabbits. One toxoid (Formalin treated for 240 h) elicited good CHO cell cytotoxicity-neutralizing titers in mice with and without adjuvant, although injection with adjuvant did result in a modest increase in antibody response (Table 1). However, toxoids produced by Formalin treatment alone increased in toxicity after the removal of Formalin and incubation at 37°C. This characteristic contraindicates the use of these toxoids as potential toxoid vaccines.

Toxin treated with Formalin plus lysine for 48 h was stable to toxic reversion after the removal of Formalin and prolonged incubation at 37°C. The immune response to this toxoid in mice was poor when adjuvant was not used. When this toxoid was used as an immunogen in combination with adjuvant (either Freund adjuvant or *N*-acetyl muramyl-L-alanyl-D-isoglutamine), good titers of CHO cell cytotoxicity-neutralizing antibody were elicited both in mice and in rabbits. These findings suggest that the Formalin-lysine toxoid preparation may have potential for use as a vaccine if a suitable adjuvant is used with the antigen. Work is now in progress to determine the effect of alum adsorption on the immunogenicity of the Formalin-lysine toxoid.

Our studies were performed by using a single preparation of purified toxin A. Subsequently, we utilized a second preparation of pure toxin A to produce Formol-derived toxoids and observed comparable results.

ACKNOWLEDGMENTS

We thank Marvin B. Rittenberg for his help in the analysis of the data and in the preparation of this manuscript. Expert technical assistance was provided by Jack Lile, John Bradley, and Linda Lewis.

This investigation was supported by Public Health Service grant IAI-14671 from the National Institute of Allergy and Infectious Diseases and by U.S. Naval Research and Development Command Research Work Units M0095.PM002.5052 and M0095.PN002.5059. R.L.F. was supported by a fellowship from the Cystic Fibrosis Foundation.

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